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14. ABSTRACT
The purpose of this proposed study is to apply genome-wide technologies to analyze ovarian cancer genome and transcriptome. In the past year, we have made substantial progress toward identifying the DNA copy number changes at a genome-wide scale and studying the molecular mechanisms of the most promising cancer-associated genes within new amplicons. Specifically, we found that SNP array analysis can provide a similar performance to profile DNA copy number alterations as digital karyotyping because of recent advances in technology refinement. Furthermore, SNP arrays would allow us to analyze more samples because of its lower cost. As a result, we have analyzed more than 40 affinity purified ovarian serous tumors and our results demonstrated that CCNE1, Notch3, Rsf-1, AKT2 and PIK3CA are among the most frequently amplified loci in high-grade serous carcinomas. Novel amplifications and sub-chromosomal deletions were also detected by the SNP array analysis. Our study is the first comprehensive analysis of the detailed amplicon profiles in purified ovarian serous tumors. In addition, we have further characterized the biological functions of the two of the commonly amplified genes, Notch3 and Rsf-1, in ovarian carcinoma. The results from this study shall provide new insight into how amplified genes contribute to survival and growth of cancer cells.

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Table of Contents

Introduction

Identification of new oncogenes and tumor suppressor genes that participate in the development of ovarian carcinoma holds great promise to develop new strategies for diagnosing and treating this devastating disease. Several elegant studies have employed gene expression profiling as the discovery tool (such as DNA-based microarray) and have identified multiple candidate markers associated with ovarian carcinomas. Several of these over-expressed genes have proven to be novel biomarkers of cancer (Hough et al., 2000). However, it is challenging to use such approach alone to distinguish the truly important genes that directly drive tumor progression from a larger number of the “passenger” genes that are co-overexpressed but lack the biological roles in tumor development. This is because gene expression is dynamic, depending on both genetic program and tumor microenvironment. In contrast, molecular genetic changes such as gene amplification and point mutation are inheritable traits as a result of clonal selection and they likely confer a growth advantage to tumor cells and propel tumorigenesis.

We hypothesize that a comprehensive analysis of ovarian cancer genome by combining genomic analysis (amplification and somatic mutation) and gene expression analysis would significantly facilitate the identification of oncogenes that directly contribute to the development of ovarian tumors. The main objective is to identify the oncogenic alterations that participate in the development of ovarian serous carcinoma, the most common and malignant type of ovarian cancer. This goal will be achieved through a parallel analysis of cancer genome and transcriptome. This approach will identify genes demonstrating increases in copy numbers of both genomic DNA and mRNA and those genes will be characterized through mutational analysis. Digital karyotyping, a technology developed in our research team, and high resolution SNP arrays permit a detailed analysis of cancer genome (Wang et al., 2002), and both techniques were employed to scan ovarian cancer genome. SAGE (serial analysis of gene expression) (Velculescu et al., 1995) and high throughput quantitative real-time PCR were performed to reveal the transcriptome in each amplified region detected by digital karyotyping. Using the above approaches, we have identified several candidate oncogenes with concurrent gene amplification and transcript/protein up-regulation and currently, we are focusing on their functional significances in tumor development.

Body

There are no substantial changes or modifications of the original statements except we also performed high density SNP array analysis on additional 43 ovarian tumor samples to complement and extend our studies. The accomplishments associated with each task outlined in the approved statement of work are detailed, point by point, in the followings. Further details can be found in the published and submitted papers (section of Reportable Outcomes).

Task 1. To identify genome-wide amplifications in ovarian serous carcinomas using Digital Karyotyping.

By the end of the 3rd year funding period, we have finished digital karyotyping libraries for serous carcinomas as originally proposed. In the mean time, we also used SNP arrays for the same purpose. This is because the technology and analysis software of high density SNP array have become mature. We have performed side-by-side comparison of digital karyotyping and high density SNP array and found the results are quite comparable between these two methods. Due to much lower cost for SNP array analysis, we are able to analyze larger number of samples. As a result, we have analyzed a total of 43 additional affinity purified ovarian serous tumors using SNP array.
Based on the SNP array and original digital karyotyping analyses, we have focused on two of the most frequently amplified regions, 11q13.2 (Rsf-1 locus) and 19p13 (Notch3 locus), for detailed mapping and functional analyses.

Task 2. To analyze genome-wide mRNA expression in the same tumors studied in AIM 1.

This task has been completed during year 1-2. Please see the previous report.

Task 3. To generate transcriptome map and compare to genomic map to identify the genes with both amplification and over-expression in the same tumor samples.

This task has been completed during year 1-2. Please see the previous report.

Task 4. To identify somatic mutations in candidate genes with both amplification and over-expression.

In the past year, we have performed mutation analysis in the kinase gene families using a high throughput platform in a series of ovarian serous tumors. We specifically focus on the kinase genes in which the mutations have been identified in human cancer and the inhibitors are available in clinical trials for other types of cancer. Once somatic mutation is identified, we will then test whether the compounds that inhibit a specific kinase pathway can abolish tumor growth in tumors that harbor the specific mutations. So far, we have performed nearly 10,000 sequencing reactions to analyze 12 kinase genes that have been reported to be amplified or mutated in human cancer in a total of 24 low-grade (MPSC) tumors and 22 high-grade serous carcinomas. We found that KRAS and BRAF are the major kinases showing mutations in low-grade tumors. High-grade carcinomas did not show mutations in those genes. In addition, three low-grade tumors demonstrated mutations in PIK3CA, a kinase that plays a central role in tumor development and PIK3CA inhibitors are available. With this preliminary result kindly supported by DoD, we are seeking for further funding resources to continue such large scale mutation analysis study. The findings generated from this comprehensive sequencing analysis will lend more insights into the etiology of ovarian cancer and will have important implications for the treatment of ovarian tumors.

Key Research Accomplishments during Year 3

- Perform high density SNP array analysis on 43 purified ovarian tumors and identify novel discrete genomic alterations.

- Perform large scale FISH analysis on seven of the most frequently amplified chromosomal loci including *CCNE1, AKT2, Notch3, Rsf-1, PIK3CA*, and *chr12p13*. Establish the database of chromosomal amplification in ovarian carcinoma.

- Functional analysis of Rsf-1 as the cancer associated genes, Identify its interaction with another chromatin remodeling protein, hSNF2H, is critical for its tumor promoting property.

- Functional analysis of Notch3, its receptor ligand, and its downstream target genes in ovarian cancer.

- Mutational analysis of kinase gene family including *PIK3CA* and *AKT2*. So far, somatic mutation was identified in *KRAS, BRAF, PIK3CA*, and *ERBB*2. Interestingly, the mutations are primarily identified in low-grade serous tumors.

Reportable Outcomes

Articles published in the 3nd year of funding period (January, 2007-current)

- Salani R, Davidson B, Fiegl M, Marth C, Müller-Holzner E, Gastl G, Huang HY, Hsiao JC, Lin HS, Wang TL, Lin BL, Shih IeM (2007) Measurement of cyclin E genomic copy number and strand length in cell-free DNA distinguish malignant versus benign effusions. *Clin Cancer Res*. 2007 Oct 1;13(19):5805-9.
- Nakayama K, Nakayama N, Wang TL, Shih IeM (2007) NAC-1 controls cell growth and survival by repressing transcription of Gadd45GIP1, a candidate tumor suppressor. *Cancer Res*. 2007 Sep 1;67(17):8058-64.
- Leary RJ, Cummins J, Wang TL, Velculescu VE (2007) *Digital karyotyping*. Nature Protocol. 2007;2(8):1973-86.
- Salani R, Kurman RJ, Giuntoli R 2nd, Gardner G, Bristow R, Wang TL, Shih IM. Assessment of TP53 mutation using purified tissue samples of ovarian serous carcinomas reveals a higher mutation rate than previously reported and does not correlate with drug resistance. *Int J Gynecol Cancer*. 2007 Aug 10; [Epub ahead of print]
- Salani R, Neuberger I, Kurman RJ, Bristow RE, Chang HW, Wang TL, Shih IeM (2007) Expression of extracellular matrix proteins in ovarian serous tumors. *Int J Gynecol Pathol*. 2007 Apr;26(2):141-6.
- Davidson B, Berner A, Trope' CG, Wang TL, Shih IeM (2007) Expression and clinical role of the bric-a-brac tramtrack broad complex/poxvirus and zinc protein NAC-1 in ovarian carcinoma effusions. *Hum Pathol*. 2007 Jul;38(7):1030-6.
- Nakayama K, Nakayama N, Jinawath N, Salani R, Kurman RJ, Shih IeM, Wang TL (2007) Amplicon profiles in ovarian serous carcinomas. *Int J Cancer*. 2007 Jun 15;120(12):2613-7
- IM Shih and T-L Wang (2007) Notch signaling, gamma secretase inhibitors and cancer therapy. *Cancer Res*. 2007 Mar 1;67(5):1879-82.

Manuscripts under review

- J J-C Sheu, J-H Choi, I Yýldýz, F-J Tsai, Y Shaul, T-L Wang, I-M Shih. (2008) The roles of hSNF2H in the tumor-promoting functions of Rsf-1. Under revision in *Cancer Research*.
- J-H Choi, J T Park, B Davidson, Patrice J. Morin, I-M Shih, and T-L Wang (2008) Jagged-1 and Notch3 juxtacrine loop regulates ovarian tumor growth and adhesion. Under review in *Cancer Research*.
- J T Park, I-M Shih, T-L Wang (2008) Notch3 promotes cancer cell survival by transcriptional upregulation of a potential oncogene, Pbx1. Under review in *PNAS*.

Grant received based on the results from this project

Individual Investigator Award, Ovarian Cancer Research Fund, 1/1/2008 to 12/31/2009

Graduate student Rebecca Busch received Provost Research Award, Johns Hopkins University

Research resource

A centralized web deposit of digital karyotyping data is initiated by the Cancer Genome Anatomy Project, NCI (http://cgap-stage.nci.nih.gov/SAGE/DKViewHome). All the sequence tags from each digital karyotyping libraries can be retrieved from this website and the browser provides bioinformatics tools to analyze the DNA copy number alterations using varying parameters, including window scales, and size and fold of alterations. Currently there are libraries deposited and in the future all the data generated from this project will be publicly available at this web link.

For SNP array database, our plan is once the manuscript is published, the raw data will be deposited onto website at Johns Hopkins University, Department of Pathology. This will be publicly assessable to general audiences.

Conclusions

Ovarian epithelial tumor is the most common type most lethal type of gynecologic malignancy. The main purpose of this project is to delineate the genomic alterations in ovarian serous tumors and identity the genes that contribute to tumor progression. The 3rd year of this project has made progress toward this goal as we have accomplished many of the tasks proposed in the timetable. Because of the low cost associated with high-density SNP array analysis, we are able to perform analysis on 43 additional purified ovarian tumors. As a result, a host of novel genomic alterations were identified.

We had performed detailed transcriptome analysis of 11q13 and 19p13 amplicons and identified *Rsf-1* and *Notch3* as the genes with most consistent gene amplification and transcript/protein up-regulation. Furthermore, survival data showed that the patients with *Rsf-1* amplification fared worse than patients without the ramped-up genes. The function of Rsf-1 and Notch3 in proliferation was also established by RNAi knock-down assays. Currently we are working on delineating the mechanism of these two oncogenic pathways in ovarian carcinoma.

Our genome scan also identified deleted regions in ovarian cancer genome. We had characterized a region with frequent homozygous deletion at 17p12. This region harbors a candidate tumor suppressor: MKK4. Function study by others as well as our work indicated that MKK4 can suppress the metastasis potential of cancer cells. Additional homozygous deletions were identified more recently by high-density SNP array analysis and currently we are pinpointing the culprit tumor suppressor within the regions with homozygous deletion.

Implications and significance of the accomplished research findings: This proposal represents a relatively comprehensive analysis of molecular genetic changes in ovarian serous tumors. Our data implies that genome-wide analysis and tools are useful in identifying novel amplified and deleted genes in ovarian cancer. These new ovarian cancer related genes may have significant impact in revealing molecular etiology of ovarian cancer and providing new targets for future development of therapeutic strategies.

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Appendices

Representative Publications in the 3rd year of project:

1. IM Shih and T-L Wang (2007) Notch signaling, gamma secretase inhibitors and cancer therapy. *Cancer Res*. 2007 Mar 1;67(5):1879-82.

2. Nakayama K, Nakayama N, Jinawath N, Salani R, Kurman RJ, Shih IeM, Wang TL (2007) Amplicon profiles in ovarian serous carcinomas. *Int J Cancer*. 2007 Jun 15;120(12):2613-7.

Notch Signaling, γ-Secretase Inhibitors, and Cancer Therapy

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Abstract

The Notch signaling pathway represents a critical component in the molecular circuits that control cell fate during development. Aberrant activation of this pathway contributes to tumorigenesis. The role of Notch in human cancer has been highlighted recently by the presence of activating mutations and amplification of Notch genes in human cancer and by the demonstration that genes in the Notch signaling pathway could be potential therapeutic targets. It has become clear that one of the major therapeutic targets in the Notch pathway are the Notch receptors, in which γ-secretase inhibitors prevent the generation of the oncogenic (intracellular) domain of Notch molecules and suppress the Notch activity. This review article summarizes the biological roles of Notch molecules in cancer development with special emphasis on the promise and challenges in applying γ-secretase inhibitors as a new line of targeted therapeutic agents. [Cancer Res 2007;67(5):1879–82]

Background

The Notch signaling pathway is evolutionarily conserved and the basic molecular players in this pathway are ligands (Delta and Jagged), Notch receptors, and the transcription factors (reviewed in ref. 1). Notch is a transmembrane heterodimeric receptor and there are four distinct members (Notch1 to Notch4) in humans and rodents. In a physiologic condition, binding of the Notch ligand to its receptor initiates Notch signaling by releasing the intracellular domain of the Notch receptor (Notch-IC) through a cascade of proteolytic cleavages by both α-secretase (also called tumor necrosis factor-α–converting enzyme) and γ-secretase (Fig. 1*A*). The released intracellular Notch-IC then translocates into the nucleus where it modulates gene expression primarily by binding to a ubiquitous transcription factor, CBF1, suppressor of hairless, Lag-1 (CSL). This binding recruits transcription activators to the CSL complex and converts it from a transcriptional repressor into an activator, which turns on several downstream effectors. The physiologic functions of Notch signaling are multifaceted, including maintenance of stem cells, specification of cell fate, and regulation of differentiation in development as well as in oncogenesis (2, 3).

In cancers, molecular genetic alterations, such as chromosomal translocation, point mutations, and chromosomal amplification at the Notch receptor loci, are the known mechanisms for constitutive activation of Notch pathway. Despite the different mechanisms, they all result in increased levels of intracellular Notch-IC. The oncogenic potential of Notch was first discovered in human T-cell acute lymphoblastic leukemia (T-ALL). While Notch1 signaling is essential for normal development of T-cell progenitors (4), constitutive activation of Notch1 signaling due to molecular genetic alterations is associated with T-ALL. For example, interstitial deletions of the extracellular portion of human Notch1 due to (7;9) chromosomal translocation are associated with ∼10% of T-ALL cases and activating point mutations of Notch1 are present in ∼50% of T-ALL cases (5, 6). Constitutive activation of nuclear factor-κB and formation of T-cell leukemia/lymphoma were observed in a Notch-IC transgenic mouse model (7), which indicates a causal role of Notch activation in T-ALL development. In non–small cell lung cancer, chromosomal translocation (15;19) has been identified in a subset of tumors, and the translocation is thought to elevate Notch3 transcription in tumors (8). In ovarian cancer, *Notch3* gene amplification was found to occur in ∼19% of tumors, and overexpression of Notch3 was found in more than half of the ovarian serous carcinomas (9). Similarly, Notch signaling activation has been shown in the development of breast cancer. In animal models, constitutively active Notch4 expression causes mammary tumors in mice (10) and Notch1-activating mutations contribute to the development of T-ALL. A recent study further shows that overexpression of activated Notch1 and Notch3 in transgenic mice blocks mammary gland development and induces mouse breast tumors (11). Overexpression of Notch3 is sufficient to induce choroid plexus tumor formation in a mouse model, suggesting a role of Notch3 in the development of certain types of brain tumors (12).

γ-Secretase as a Key Mediator of Notch Signaling

Because the Notch-IC signaling plays an important role in cancer development, it is plausible that targeting the Notch signaling steps, including receptor/ligand binding, release of Notch-IC, interaction of Notch-IC and downstream targets, as well as Notch-IC protein stability, can have antitumor effects (13, 14). Currently, one of the emerging approaches for blocking Notch signaling is to suppress the proteolytic step that leads to the generation of intracellular Notch-IC (Fig. 1*B*). On ligand binding, Notch receptors undergo a series of programmed proteolytic events, first by α-secretase at the extracellular surface, which leads to liberation of the extracellular fragment, and then by intramembranous cleavage mediated by γ-secretase. Notch-IC is then released from the inner surface of cell membrane and is translocated into nucleus where it activates transcription of the target genes. The proteolytic events in Notch signaling activation are comparable with the processes involving amyloid precursor protein (APP) cleavage (Fig. 1*B*), in which sequential cleavages by β-secretase and γ-secretase release the amyloid β-peptide (the precursor of amyloid plaques found in the brain of Alzheimer's disease).

γ-Secretase is a large protease complex and is composed of a catalytic subunit (presenilin-1 or presenilin-2) and accessory subunits (Pen-2, Aph1, and nicastrin). All these subunits contain transmembrane domains and thus they are membrane proteins. The pivotal role of γ-secretase in the Notch activation cascade has been well shown in an elegant knockin experiment, showing that introduction of a single point mutation near the transmembrane cleavage site in Notch1 molecules results in an embryonic lethality in mice, which is similar to the effects observed in Notch1

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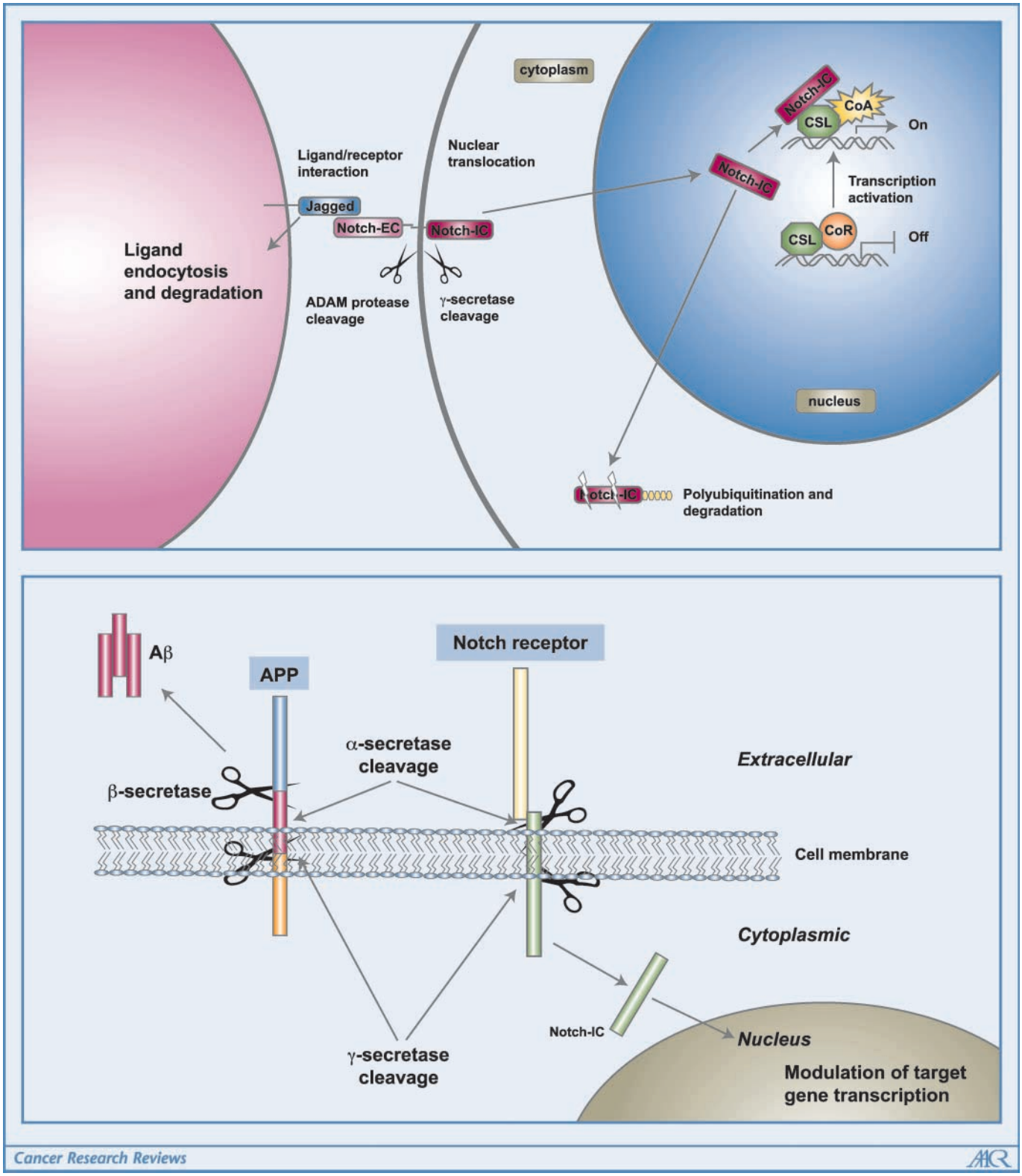

Figure 1. An overview of Notch signaling and proteolytic processing. *Top,* Notch receptor is a cell surface protein. Interaction with the Notch ligand, such as Jagged, initiates proteolytic cleavage at the extracellular site by α-secretase followed by cleavage at the intracellular site by γ-secretase, resulting in the release of Notch-IC from the cytoplasmic side of the cell membrane. Notch-IC is then translocated into the nucleus where it interacts with CSL and recruits coactivators (CoA) to form a transcription-activating complex. Notch-IC can be polyubiquitylated and targeted for degradation in a proteasome-dependent manner. However, the location of degradation is currently not clear. *Bottom,* the proteolytic events involving Notch receptor and APP are similar. APP is an integral membrane protein and is cleaved at the extracellular site by β-secretase followed by γ-secretase to release Aβ peptides. Aβ is the major constituent of amyloid plaques and its accumulation is thought to play a central role in Alzheimer's disease. APP can be also cleaved by α-secretase and γ-secretase, which leads to liberation of P3 peptides with unknown functions.

knockout (15). Furthermore, the presenilin-1–deficient and presenilin-1/presenilin-2 double knockout mice had a marked decrease in Notch-IC generation (16, 17).

Over the past decades, inhibitors for γ-secretase have been actively investigated for their potential to block the generation of Aβ peptide that is associated with Alzheimer's disease (18). Because γ-secretase inhibitors are also able to prevent Notch receptor activation, several forms of γ-secretase inhibitors have been tested for antitumor effects. First, an original γ-secretase inhibitor, IL-X (cbz-IL-CHO), was shown to have Notch1-dependent antineoplastic activity in Ras-transformed fibroblasts. More recently, tripeptide γ-secretase inhibitor (z-Leu-leu-Nle-CHO) was reported to suppress tumor growth in cell lines and/or xenografts in mice from melanoma and Kaposi sarcoma (19). Treatment with dipeptide γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) also resulted in a marked reduction in medulloblastoma growth and induced G_0-G_1 cell cycle arrest and apoptosis in a T-ALL animal model (20, 21). Another γ-secretase inhibitor, dibenzazepine, has been shown to inhibit epithelial cell proliferation and induce goblet cell differentiation in intestinal adenomas in $Apc^{-/-}$ (min) mice (22). More recently, functional inactivation of Notch3 either by tripeptide γ-secretase inhibitor or Notch3-specific small interfering RNA results in suppression of cell proliferation and induction of apoptosis in the tumor cell lines that overexpressed Notch3 but not in those with minimal amounts of Notch3 expression (9). Futhermore, a phase I clinical trial for a Notch inhibitor, MK0752 (developed by Merck, Whitehouse Station, NJ), has been launched for relapsed or refractory T-ALL patients and advanced breast cancers.[1] As discussed above, Notch signaling and APP metabolism are triggered by the similar proteolytic process; it is foreseeable that γ-secretase inhibitors that are currently tested in clinical trials for Alzheimer's disease may be applicable to treat neoplastic diseases, especially those tumors known to harbor constitutive Notch activation. Besides the evidence of γ-secretase inhibitors in directly inactivating Notch signaling on cancer cells, γ-secretase inhibitors may also suppress angiogenesis in solid tumors by interfering in the cross-talk between the tumor and vasculature through the Notch signaling (23).

The exciting studies summarized above strongly suggest a potential clinical application of γ-secretase inhibitors in cancer therapeutics. However, one of the major challenges on the way toward this goal is the untoward side effects associated with the inhibitors, especially the cytotoxicity in the gastrointestinal tract (24), which can be exacerbated by conventional chemotherapeutic drugs. Therefore, balancing efficacy and toxicity of γ-secretase inhibitors must be considered in future clinical applications. The possible mechanisms underlying the unwanted cytotoxicity are multifactorial. First, Notch signaling pathway is known to widely participate in cellular physiology in normal tissues, including hematopoiesis and maintenance of arterial smooth muscle (25); therefore, it is plausible that inactivation of γ-secretase may lead to dysfunction of vital organs. Second, it is likely that γ-secretase inhibitors do not exclusively target the Notch signaling pathways. This is because γ-secretase has many substrates in addition to Notch receptors, such as several Notch ligands, ErbB4, syndecan (an extracellular matrix), and CD44 (1). Additionally, γ-secretase inhibitors may target proteases other than γ-secretase. As proteases participate in a wide variety of cellular functions, γ-secretase inhibitors may have other widespread adverse effects *in vivo*. Some of the concerns of the nonselectivity of the inhibitors will be addressed based on the results of the ongoing clinical trials. Nevertheless, it may prove possible to identify a therapeutic window, in which partial inhibition of γ-secretase is sufficient to suppress Notch signaling in cancer cells, whereas the dosage will not significantly affect the functions in normal tissues. It is thought that the differential killing between cancer and normal cells can be exaggerated in treating those tumors with constitutive Notch activation, in which cancer cells are "addicted" to the Notch signaling.

Implications and Future Directions

The findings discussed above have at least two major biological and clinical implications. First, like wingless (wnt) and Hedgehog (shh), the Notch signaling pathway is important in controlling both developmental processes and tumorigenesis. Tumor cells sabotage the Notch signaling pathway for tumor initiation and/or progression through constitutive activation by ways of chromosomal translocation, point mutation, gene amplification, and other epigenetic events. Second, recent studies suggest that one of the most promising targets in inactivating the Notch signaling is γ-secretase complex, the molecular switch for Notch signaling activation. Recently, there has been an increased enthusiasm in targeting this pathway using γ-secretase inhibitors for new cancer therapeutics because accumulating preclinical studies have shown that γ-secretase inhibitors hold promise as a new target-based therapy for those tumors with Notch activation. However, before Notch-based therapy becomes a reality, future studies should primarily focus on the issues of target specificity and address the possible side effects that may affect cancer patients who receive this new treatment regimen. Furthermore, the clinical promise of γ-secretase inhibitors in cancer therapeutics depends on careful correlation studies between the molecular genetic alterations in the *Notch* gene (e.g., mutations and gene amplification) and clinical response to γ-secretase inhibitors. To maximize the therapeutic effects (together with conventional therapeutics) and minimize the adverse side effects in cancer patients, it is essential to show the "tumor dependency" of Notch activation experimentally and clinically. Despite several challenges on the way, it is expected that in the coming years, there will be substantial efforts in identifying new specific γ-secretase inhibitors and in opening new clinical trials to test the potential of this new line of cancer therapeutic agents.

Acknowledgments

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[1] http://www.clinicaltrials.gov/ct/show/NCT00100152

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Amplicon profiles in ovarian serous carcinomas

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Ovarian serous carcinoma is the most common and lethal type of ovarian cancer and its molecular etiology remains poorly understood. As an ongoing effort to elucidate the pathogenesis of ovarian serous carcinomas, we assessed the DNA copy number changes in 33 high-grade serous carcinomas and 10 low-grade serous tumors by using a genome-wide technique, single nucleotide polymorphism array, performed on affinity-purified tumor cells from fresh surgical specimens. Compared to low-grade tumors, high-grade serous carcinomas showed widespread DNA copy number changes. The most frequent alterations were in loci harboring candidate oncogenes: *cyclin E1 (CCNE1)*, *AKT2*, *Notch3* and *PIK3CA* as well as in novel loci, including 12p13, 8q24, 12p13 and 12q15. Seven amplicons were selected for dual color fluorescence *in situ* hybridization analysis in ~90 high-grade serous carcinomas and 26 low-grade serous tumors, and a high level of DNA copy number gain (amplification) was found in *CCNE1*, *Notch3*, *HBXAP/Rsf-1*, *AKT2*, *PIK3CA* and chr12p13 occurring in 36.1%, 7.8%, 15.7%, 13.6%, 10.8% and 7.3% of high-grade serous carcinomas. In contrast, we did not observe high level of *ERBB2* amplification in any of the samples. Low-grade tumors did not show DNA copy number gain in any of the loci, except in 2 (8%) of 24 low-grade tumors showing low copy number gain in the *Notch3* locus. Taken together, our results provide the first comprehensive analysis of DNA copy number changes in highly pure ovarian serous carcinoma. These findings may have important biological and clinical implications.

Key words: oncogene; amplification; ovarian cancer; karyotyping; genetics

DNA copy number alterations, including amplification, deletion and aneuploidy in chromosomes, are the hallmarks of neoplasia.[1] Amplification of chromosomal regions plays a critical role in tumor development. Increase in copy number of oncogenes promotes initiation and progression of a variety of solid tumors, while amplification of genes that modify or detoxify chemotherapeutic drugs can cause drug resistance and is associated with tumor recurrence.[2,3] Well-known amplified oncogenes include *c-myc*, *ERBB2*, *EGFR*, *AKT2*, *CCND1* and *cyclin E1 (CCNE1)*.[4] Studies on these genes not only have provided new insights on how cancer develops but also have significant translational implications. For example, *ERBB2* and *EGFR* are the molecular targets for the humanized antibodies Trastuzumab (Herceptin) and Matuzumab respectively, which are used in the treatment of breast and lung cancer. The completion of the human genome database has accelerated cancer genome study, as it provides precise and detailed maps to facilitate localization of genes potentially important in cancer. Several genome-wide technologies, including digital karyotyping, array comparative genomic hybridization (CGH), ROMA and single nucleotide polymorphism (SNP) array, have recently been developed and applied to explore the molecular genetic landscape of cancer in the postgenomic era.[5]

Epithelial ovarian cancer is the most lethal gynecologic neoplastic disease and one of the leading causes of cancer mortality in women. It comprises a diverse group of tumors, and among them, serous carcinoma is the most common and the most lethal type. Among serous neoplasms, 70% are high-grade and 30% are low grade tumors (including invasive low-grade carcinoma and its precursor lesion, serous borderline tumor). Despite considerable efforts aimed at elucidating the molecular etiology of ovarian serous carcinoma, its pathogenesis is still largely unknown. Although many genes have been previously reported to be amplified and deleted in ovarian cancer,[6–13] the overall profile of DNA copy number alterations in high-grade serous carcinomas has not been well described. One of the reasons is that most previous studies have combined different histological types of ovarian cancer, and therefore the specific alterations in the different histological types of ovarian serous carcinoma are not known. Furthermore, in most studies the tumor samples were not enriched by microdissection or affinity purification, which could also have obfuscated the interpretation of the results. Therefore, in this study, we analyzed a total of 43 affinity-purified ovarian serous tumors by using 10K SNP array to identify the chromosomal regions that show the most frequent DNA copy number changes. SNP array was used because it has been recently demonstrated to be feasible and robust and, most important, this technology provides high resolution which facilitates detection of DNA copy number changes.[14–16] In this study, we focused on amplified regions because it appeared more feasible to validate amplicons rather than deletions in regions in which the DNA copy number changes were usually subtle (from 2 to 1 or 0 copy). Our results demonstrate that *CCNE1*, *Notch3*, *HBXAP/Rsf-1, AKT2* and *PIK3CA* are among the most frequently amplified loci in high-grade serous carcinomas. In addition, novel amplifications and subchromosomal deletions were also detected by the SNP array analysis.

Material and methods

Tumor specimens

For SNP arrays, tissue samples from 33 high-grade ovarian serous carcinomas and 10 low-grade ovarian serous tumors (8 serous borderline tumors and 2 low-grade serous carcinomas) were freshly collected from the Department of Pathology at the Johns Hopkins Hospital from 2003 to 2006. The 33 high-grade serous carcinomas were all Stage III or IV and 5 of them were recurrent tumors. In addition, 15 normal ovaries served as controls. Tumor cells were affinity purified by anti-EPCAM-conjugated beads as previously described.[5,13] The acquisition of the anonymous tissue specimens for this study was approved by the Johns Hopkins Institutional Review Board.

Single nucleotide polymorphism array

SNPs were genotyped using 10K arrays (Affymetrix, Santa Clara, CA) in the Microarray Core Facility at the Dana-Farber

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TABLE I – BAC CLONES USED FOR FISH STUDY

Gene name	BAC clone name	Location
PIK3CA	RP11-245C23 and RP11 -355N16	3q26.32
Rsf-1 (HBXAP)	RP11-45G10 and RP11-1107J12	11q14.1
Chr12p	RP11-62E21 and RP11-35017	12q13.32
ERBB2	RP11-94L15 and CTD-2019C10	17q12
Notch3	RP11-937H1 and RP11-319O10	19p13.12
Cyclin E1	RP11-345J21 and CTD-3005A16	19q12
AKT2	RP11-639F21 and CTC-425O23	12q13.2
Control	RP11-127K18 and RP11-629A22	2q11.2

Cancer Institute (Boston, MA). A detailed protocol is available at the Core center webpage (http://chip.dfci.harvard.edu/lab/services.php). Briefly, genomic DNA was cleaved with the restriction enzyme XbaI and ligated with linkers, followed by PCR amplification. The PCR products were purified and then digested with DNaseI to a size ranging from 250–2,000 bp. Fragmented PCR products were then labeled with biotin and hybridized to the array. Arrays were then washed on the Affymetrix fluidics stations. The bound DNA was then fluorescently labeled using streptavidin–phycoerythrin conjugates and scanned using the Gene Chip Scanner 3000.

dChip software (version 1.3) was used to analyze the SNP array data as described previously.[7,8] Data were normalized to a baseline array with median signal intensity at the probe intensity level using the invariant set normalization method. A model-based (PM/MM) method was employed to obtain the signal values for each SNP in each array. Signal values for each SNP were compared with the average intensities from 15 normal samples. To infer the DNA copy number from the raw signal data, we used the Hidden Markov Model,[7] based on the assumption of diploidy for normal samples. Mapping information of SNP locations and cytogenetic band were based on curation of Affymetrix and University of California Santa Cruz hg15. A cutoff of >3.5 copies in more than 3 consecutive SNPs was defined as amplification.

Fluorescence in situ *hybridization*

BAC clones were purchased from Bacpac Resources (Children's Hospital, Oakland, CA) or Invitrogen (Carlsbad, CA). The BAC clones used in this study are summarized in Table I. Bac clones (RP11-127K18 and RP11-629A22), located at Chr2q11.2, were used to generate the reference probes. The method for fluorescence *in situ* hybridization (FISH) has been detailed in previous reports.[3] The hybridization signals were counted by 2 individuals. The signal ratio of experimental probe/reference probe greater than 2 is considered as gain, and the signal ratio of experimental probe/reference probe greater than 3 is considered as high-fold amplification.

Results

Wide spread DNA copy number changes in high-grade serous carcinomas

The results of DNA copy number changes in all tumor specimens are shown in Figure 1. Compared to low-grade serous tumors (serous borderline tumors and invasive low-grade micropapillary serous carcinomas), the high-grade serous carcinomas demonstrated DNA copy number alterations in the majority of chromosomes. The number and amplitude of changes were much higher in high-grade than in low-grade serous tumors. The DNA copy number alterations in high-grade serous carcinomas involved gain or loss of discrete subchromosomal regions, chromosomal arms and whole chromosomes. In contrast, the low-grade tumors were characterized by a relatively "flat" chromosomal landscape with only a few chromosomal losses and low copy number gains. On the basin SNP array analysis, the regions with high prevalence of copy number gain (>3.5 copies) in high-grade serous carcinomas included regions that contain known putative oncogenes *CCNE1* at chr19q13.1 (33.3%), *AKT2* at chr19q13.2 (27.3%), *Notch3* at chr19p13.12 (21.2%) and *PIK3CA* at chr3q26.32 (9.1%), and novel amplicons 12p13, 8q24, 12p13 and 12q15. Interestingly, the *HBXAP*/*Rsf-1* region at chr11q13.5, which was previously reported to be amplified in ovarian serous carcinomas,[10] was found rarely amplified in this study, as *HBXAP*/*Rsf-1* amplification was only identified in 1 of 33 in this series of tumor samples. This is likely due to the relatively smaller sample size in the SNP array analysis, since FISH analysis in a larger number of specimens in the current study demonstrated an amplification frequency of 15.7%, close to previous reports.[10,11] Another well-known amplified oncogene in breast carcinoma, *ERBB2*, which is located at chr17q12, was not found to be amplified at high levels in any of the high-grade serous carcinomas and low-grade serous tumors.

In addition to the genomic amplification, there were many chromosomal regions demonstrating decrease in DNA copy number in small discrete regions or the whole arm, notably in chr8p, chr16, chr17p and chrX.

Dual-color FISH analysis of CCNE1, Notch3, HBXAP/Rsf-1, AKT2 and PIK3CA amplification in high-grade serous carcinomas

To independently validate the results of SNP arrays, we performed FISH on 98 paraffin-embedded high-grade ovarian tumors and 26 low-grade serous tumors. FISH was used because the method allowed a direct count of the DNA copy number in tumor cells. FISH probes were designed to hybridize *CCNE1*, *Notch3*, *AKT2*, *HBXAP*/*Rsf-1* and *PI3KCA* loci (Fig. 3). In addition, the frequency of *ERBB2* amplification in serous carcinoma was also determined. Chr12p13 was also analyzed because it was the most common amplicon in which the amplified oncogene(s) was unknown. In this study, we used the chr2q11.2 as the reference locus for FISH because the DNA copy number in this specific chromosomal region was relatively constant among the tumors (Fig. 2) that facilitated the quantification of the DNA copy number in the targeted loci. Using this approach, we demonstrated that *CCNE1* was amplified at a high amplitude (>3-fold in ratio of experimental probe *versus* reference probe) in 36.1%, *Notch3* in 7.8%, *AKT2* in 13.6%, *HBXAP*/*Rsf-1* in 15.7%, *PIK3CA* in 10.8% and *chr12p13* in 7.3% of high-grade carcinomas (Table II). Amplification in at least one of the aforementioned genes was found in 64% of cases. An increased DNA copy number was not observed for any loci in low-grade tumors except 2 cases showing *Notch3* low copy number gain (<3 copy ratio). Interestingly, consistent with the above SNP array analysis, this large-scale FISH analysis did not detect high levels of *ERBB2* amplification in any high-grade or low-grade serous tumors.

To determine whether the amplifications of CCNE1, AKT2, Notch3, HBXAP/Rsf-1, PIK3CA and chr12p13 loci were independent or related to each other, we performed Fisher's exact test to determine the significance of correlation. The results in Table III demonstrated that *CCNE1* amplification was significantly correlated with *AKT2* amplification ($p = 0.014$), *Notch3* amplification ($p = 0.042$) and chr12p13 amplification ($p = 0.022$). In addition, there was a marginal correlation between *AKT2* and chr12p13 amplifications ($p = 0.049$).

Discussion

Serous ovarian carcinoma represents ~60% of ovarian epithelial carcinomas. It is one of the most lethal malignancies in women, with a 5-year survival rate less than 30%. This dismal outcome is in part due to the lack of known molecular genetic markers that can be potentially targeted for therapy. The most common genetic alteration known to date in high-grade serous carcinoma is p53 mutation, which occurs in >50% of tumors[17,18] (Salani, unpublished results). In this study, we explored the DNA copy number alterations in ovarian serous carcinoma and identified the gene amplification profiles and subchromosomal deletions in ovarian serous tumors.

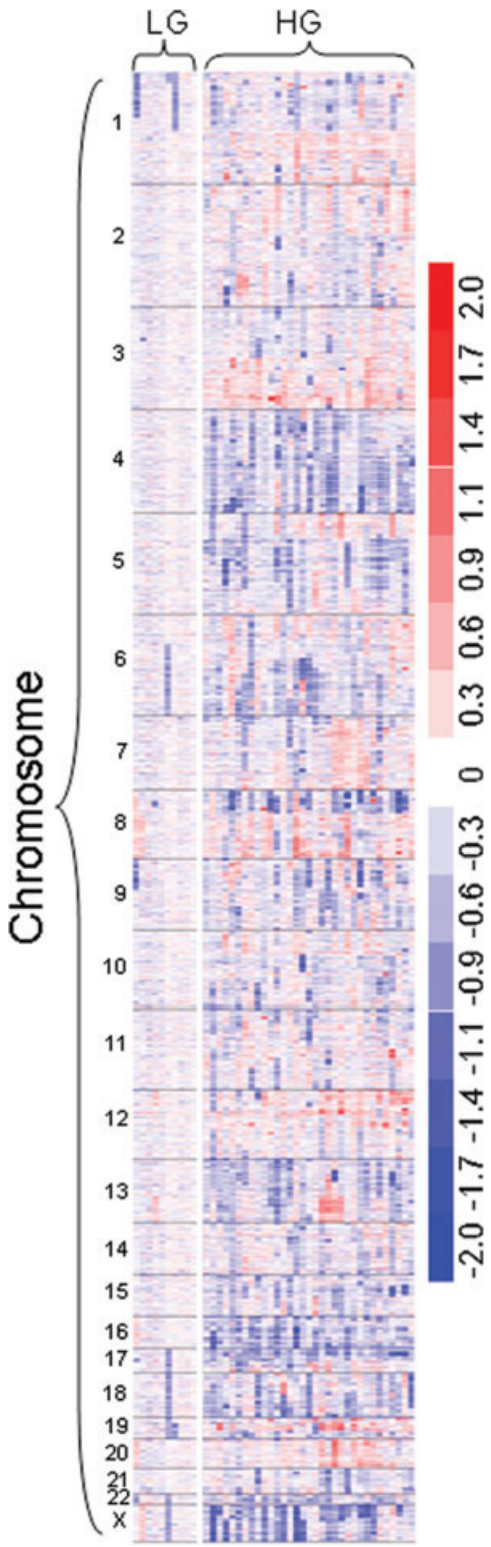

FIGURE 1 – Genome-wide distribution of DNA copy number changes in low-grade and high-grade ovarian serous carcinomas. Each column represents an individual tumor sample. DNA copy number changes are represented as pseudocolor gradients corresponding to the folds of increase (red boxes) and decrease (blue boxes), as compared to pooled normal samples. Compared to low-grade tumors, high-grade carcinomas demonstrate diffuse and sometimes, discrete DNA copy number gain (red boxes) and loss (blue boxes) in many of the chromosomes. Chromosomal numbers are shown in left column.

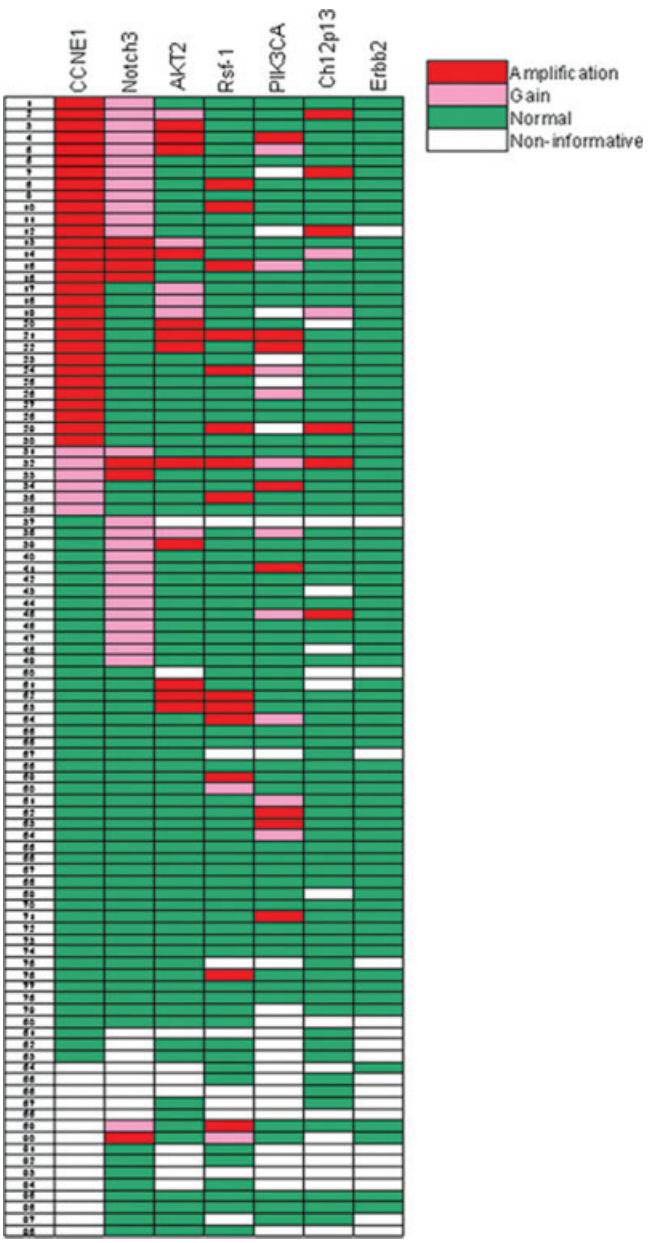

FIGURE 2 – Summary of fluorescence *in situ* hybridization (FISH) results of chromosomal regions harboring CCNE1, AKT2, Notch3, Rsf-1, PIK3CA, ERBB2 and a locus at chr12p13. A total of 98 high-grade serous carcinomas (case numbers shown in the left column) were evaluated using two-color FISH. Red boxes: amplification >3 folds (ratio of target gene signals *versus* reference gene signals greater than 3); pink boxes: DNA copy number increase between 2 and 3 folds; green boxes: normal (DNA copy ratio <2 fold); empty boxes: noninformative cases because either tumor cells are not available or FISH signal is not detectable in tissue sections. Chr2q11.2 was used as the reference locus for FISH because the DNA copy number in this specific chromosomal region was relatively constant among the tumors.

Compared to previous studies using conventional and array CGH,[19–23] the current study utilized 4 distinctly new approaches in the analysis of DNA copy number changes in ovarian cancer. First, we analyzed only serous carcinoma and did so in a relatively large number of cases. Indeed, the current analysis is to the best of our knowledge the largest study of DNA copy number alterations in ovarian serous carcinomas. Second, we used affinity-purified tumor cells directly isolated from surgical specimens for SNP

TABLE II – SUMMARY OF FISH RESULTS FOR OVARIAN SEROUS TUMORS

	Cyclin E1	*Notch3*	*AKT2*	*Rsf-1*	*PIK3CA*	Chr12p13	*ERBB2*
HG							
Normal	47 (56.6)	57 (63.3)	70 (79.5)	73 (82.1)	56 (75.7)	74 (90.2)	79 (100)
Gain	6 (7.2)	26 (28.9)	6 (6.8)	2 (2.2)	10 (13.5)	2 (2.4)	0 (0)
Amp	30 (36.1)	7 (7.8)	12 (13.6)	14 (15.7)	8 (10.8)	6 (7.3)	0 (0)
Total	83	90	88	89	74	79	82
LG							
Normal	26 (100)	22 (91.7)	24 (100)	25 (100)	20 (100)	23 (100)	24 (100)
Gain	0	2 (8.3)	0	0	0	0	0
Amp	0	0	0	0	0	0	0
Total	26	24	24	25	20	23	24

Amp, amplification. Values in parantheses indicate percentage of cases.

TABLE III – CORRELATION OF DIFFERENT AMPLICONS IN OVARIAN HIGH-GRADE SEROUS CARCINOMAS

	Cyclin E	*Notch 3*	*AKT2*	*Rsf-1*	*PI3KCA*	*Chr12p13*
Cyclin E		0.042	0.014	0.386	0.586	0.022
Notch 3	0.042		0.415	1.000	1.000	0.052
AKT2	0.014	0.415		0.741	0.335	0.049
Rsf-1	0.386	1.000	0.741		0.502	0.653
PI3KCA	0.586	1.000	0.335	0.502		0.291
Chr12p13	0.022	0.052	0.049	0.653	0.291	

The number in each box indicates the *p*-value based on the Fisher exact test.

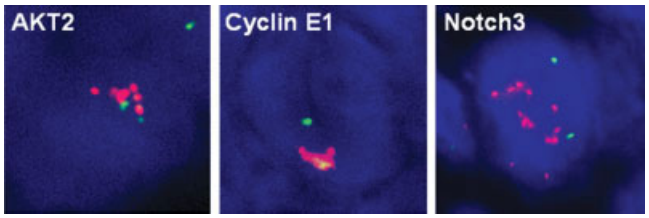

FIGURE 3 – Examples of two-color FISH for amplification in AKT2, Cyclin E1 and Notch3 loci. The numbers of red signals that represent the target gene in each case are more than those of the green signals (control probes). The nuclei are counterstained with DAPI (blue).

arrays. We believe that this approach will allow us to accurately measure the DNA copy number changes. Third, we analyzed high-grade (conventional) serous carcinomas and low-grade serous tumors separately, providing a new dimension to the molecular signatures of ovarian serous carcinoma. Fourth, in addition to SNP array analysis, we performed FISH on an independent set of tumor tissues. Our FISH results further validated the amplicon profiles based on SNP array analysis.

Using these approaches, we were able to characterize detailed amplicon profiles of ovarian serous carcinomas. It has been proposed that both high-grade and low-grade ovarian serous tumors develop along distinct molecular pathways.[24,25] Our results showing marked DNA copy number alterations in high-grade carcinoma when compared to low-grade tumors are consistent with the conclusion based on previous works using the CGH approach.[23,26] The widespread changes in DNA copy number in high-grade carcinoma suggest a high level of chromosomal instability in this type of tumor, while low-grade tumor is relatively stable. Previous studies have shown that low-grade tumors rarely harbor p53 mutation but have frequent ERBB2/KRAS/BRAF mutations.[13,27] In contrast, high-grade carcinomas are characterized by frequent p53 mutations but only rarely harbor ERBB2/KRAS/BRAF mutations. These results, together with the current findings, suggest significant different genetic/genomic profiles for these 2 types of serous ovarian serous carcinoma and further support the dualistic model of ovarian serous tumorigenesis.

Of the different amplified chromosomal regions in high-grade serous carcinomas, the most frequently amplified chromosomal loci were *CCNE1*, *Notch3*, *HBXAP*/*Rsf-1*, *AKT2* and *PIK3CA*. We did not observe amplification of the *ERBB2* locus or detect an increase in its DNA copy number by both SNP array and FISH analyses. The only sample in which we ever have *ERBB2* amplification is the SKOV3 cell line based on digital karyotyping analysis.[5] Our results suggest that *ERBB2* amplification is probably a rare event in high-grade ovarian serous carcinoma. *CCNE1* is the most commonly amplified gene in high-grade ovarian serous carcinomas based on both SNP array and FISH analyses. Amplification of *CCNE1* has been reported in several human cancers, including ovarian carcinoma.[28,29] Furthermore, *CCNE1* expression is correlated with tumor progression and predicts a poor outcome in patients with ovarian carcinoma.[6,30] *Notch3* amplicon has been recently reported in ovarian serous carcinomas based on fine mapping of the chr19p13.12 amplicon from high-grade serous carcinomas using a part of the SNP array results reported here.[16] *HBXAP*/*Rsf-1* functions as a chromatin remodeling protein by partnering with hNSF2H. The resulting complex plays an important role in a variety of cellular functions. *HBXAP*/*Rsf-1* gene has been identified as an amplified gene in ovarian cancer by digital karyotyping,[10] and its amplification was found to be associated with a shorter overall survival in patients with high-grade ovarian serous carcinoma. The PIK3CA–AKT2 signaling pathway regulates diverse cellular functions in response to extracellular milieu. Increased gene copy number of both genes has been found in lung, pancreatic, ovarian, cervical, head and neck carcinomas. Activation of the PIK3CA–AKT2 pathway either by gene amplification or by activating somatic point mutations has been implied to contribute to tumor development. Amplification of these genes may have biological and clinical significance. For example, expression of *AKT2* and *PIK3CA* genes is required for cell growth and survival in cancer cells that amplify or overexpress *AKT2* and *PIK3CA* genes.[31,32] These amplified genes may serve as the molecule targets for future therapeutic strategies in ovarian cancer.

SNP array analysis provides a whole-genome view of DNA copy number alterations in tumor specimen. Among the loci that we identified using both SNP array and FISH analyses, *CCNE1*, *PIK3CA* and chr12p13 showed closer call of amplification, while *NOTCH3* and *AKT2* had a wider deviation of amplification frequency between these 2 different techniques. Several factors could attribute to this discrepancy, including sampling differences between the 2 different analyses, different normalization methods used by each method, relatively subjective call in FISH analysis *etc*.; thus it is advisable to validate DNA copy number alteration results by at least 2 independent methods.

Some genes were found to be coamplified in the same tumor. For example, *CCNE1* frequently coamplifies with *AKT2*, *Notch3* and chr12p13. Coamplification of *CCNE1* and *AKT2* probably occurs because both genes are located in the same chr19q arm which is amplified. Likewise, coamplification of *CCNE1* and chr12p13 may be due to an unbalanced translocation followed by an amplification event. Alternatively, coamplification can be due to tumor evolution by selecting for clones with coamplification of

the genes that provide the cells with growth advantages. Whether these genes cooperate molecularly to propel tumorigenesis in ovarian cancer remains to be investigated. Besides the known candidate oncogenes, we also identified several chromosomal regions that are frequently amplified in ovarian cancer but do not harbor any known (potential) oncogenes. For example, chr12p13 was found to be amplified in 7.3% of high-grade serous tumors. Future studies are required to identify the novel gene(s) within a new amplicon. However, it is important to note that DNA copy number alteration does not necessarily indicate that the amplification plays a causal role in tumor development.

In conclusion, our SNP array and FISH analyses have shown what the prevalence of specific amplicons is in ovarian serous carcinomas. Our findings suggest that *CCNE1*, *Notch3, AKT2*, *HBXAP*/*Rsf-1* and *PIK3CA* are frequently amplified potential oncogenes in high-grade serous carcinomas. In contrast to high-grade carcinoma, low-grade serous tumors did not show high copy number gain, a finding that supports the view that high-grade serous carcinoma is characterized by pronounced chromosomal instability. These results shed light on the molecular features of ovarian carcinoma and provide candidates for molecular targeting in the treatment of ovarian cancer.

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